

【国際公開パンフレット】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
17 October 2002 (17.10.2002)

PCT

(10) International Publication Number
WO 02/080778 A1

- (51) International Patent Classification: A61B 10/00 (31) Designated States (national): AE, AG, AI, AM, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GR, GU, HK, HR, HU, ID, IL, IN, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MY, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.
- (21) International Application Number: PCT/US02/11003 (32) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SI, SZ, TZ, UG, ZM, ZW); European patent (AM, AZ, BY, BG, CZ, DE, DK, EC, ES, FI, FR, GB, GR, HU, IT, LU, MC, NL, PT, SE, TR); OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NI, NN, TD, TG).
- (22) International Filing Date: 4 April 2002 (04.04.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Date: 09/05/2001 9 April 2001 (09.04.2001) US
- (71) Applicant: FLUORO PROBE, INC. (USAUS); 1211 Alameda Boulevard, Oceanside, CA 92118 (US).
- (72) Inventor: LAUKEN, George, A.; 1211 Alameda Boulevard, Oceanside, CA 92118 (US).
- (74) Agent: LEARN, Jose, M.; Gray Cary Ware & Freidenrich LLP, Suite 1100, 4315 Executive Drive, San Diego, CA 92121-2133 (US).
- Published with international search report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/080778 A1

(54) Title: METHOD FOR VIEWING TUMOR TISSUE LOCATED WITHIN A BODY CAVITY

(57) Abstract: Methods are provided for in vivo detection of diseased tissue in a subject, such as tumor tissue located in a body cavity, by administering to the subject a biologically compatible fluorescent targeting construct that binds to or is specifically taken up by the diseased tissue. The observer directly views fluorescence emanating from the fluorescent targeting construct bound to or taken up by the diseased tissue upon irradiation of the targeting construct with excitation light having at least one wavelength in the range from 401 nm to about 495 nm, but preferably lacking light having a wavelength above about 500 nm, so as to determine the location and/or surface area of the diseased tissue in the subject. Since excitation wavelengths do not penetrate through tissue, as in the practice of near IR diagnostics, the diseased or abnormal tissue is exposed to the excitation light either surgically or by means of an endoscopic device. Preferably a filter is used to filter out any wavelengths in the excitation light greater than about 500 nm.

WO 02/080778

PCT/US02/11003

**METHOD FOR VIEWING TUMOR
TISSUE LOCATED WITHIN A BODY CAVITY**

FIELD OF THE INVENTION

The present invention relates to methods for viewing the state of a body cavity or an internal organ of a mammalian body. More particularly, the invention relates to a method for detecting tumor tissue at an interior body site using a fluorescent targeting construct excited by light in the visible light range.

RELATED APPLICATIONS

This application is a Continuation-In-Part application of copending U. S. Patent Application Serial No. 09/362,805, filed July 28, 1999, which is a Continuation-In-Part application of copending U.S. Patent Application Serial No. 09/173,190, filed October 15, 1998, entitled "Method For Viewing Diseased Tissue Located Within A Body Cavity," each of which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Many solid and liquid substances naturally emit fluorescent radiation when irradiated with ultraviolet light. However, the radiation may fall within wide wavelength bands of low intensity. In the case of many natural objects, observations are partially obscured by natural fluorescence emanating simultaneously from many different compounds present in the sample under examination. In imaging devices such as microscopes, therefore, it is known to employ a filter for a selected UV wavelength band to screen out undesired fluorescence emanating from the object under observation.

In medical applications, a similar difficulty arises because both tumors and healthy tissue fluoresce naturally, albeit at different wavelengths. Consequently, when UV-activated fluorescence is used to detect tumors against a background of healthy tissue, identification of tumors is difficult. However, unlike most other cells of the body, tumor cells may possess a natural ability to concentrate and retain

WO 02/080778

PCT/US02/1003

2

hematoporphyrin derivative dyes. Based upon this discovery, a technique was developed wherein a hematoporphyrin derivative fluorescent dye is administered and allowed to concentrate in a tumor to be examined to increase the fluorescence from the tumor as compared with that of healthy background tissue. Hematoporphyrin dyes fluoresce within a fluorescence spectrum between 610 and 700 nm, a spectrum easy to detect. However, the natural fluorescence from healthy cells is still much more intense than that from the dyes, and has a broader fluorescence spectrum. Thus, the use of fluorescent dyes in diagnosis of tumors has not been wholly successful.

In endoscopic systems, it is also known to irradiate an internal organ with visible radiation to obtain a visible image and then to apply to the internal organ a fluorescent dye that concentrates in tumors over a period of time. The dye is allowed to concentrate, and then the internal organ is irradiated with excitation radiation for the dye to obtain a second fluorescent image. A body part having abnormal or diseased tissue, such as a cancer, may be identified by comparing an image produced by visible radiation of the internal organ with the image produced by fluorescence. To aid in visualizing the images received, endoscopic systems commonly utilize a television camera attached to a fiber optic scope having an optical guide fiber for guiding a beam from an external radiation source to the internal organ, and another optical guide fiber for transmitting a fluorescent image of the affected area to a television monitor for viewing. These two approaches are combined in a method of the type disclosed in U.S. Patent 4,821,117, wherein a fluorescent dye is applied to an object to be inspected, allowed to concentrate in the tumor, and the affected site is then alternately irradiated with visible light and with radiation at the excitation wavelength of the fluorophore. Images of the object obtained independently by visible and fluorescent light using a TV camera are stored in memory, and are simultaneously displayed in a television monitor to visually distinguish the affected area of the body part from the healthy background tissue.

In another type of procedure, such as is described in U. S. Patent No. 4,786,813, a beam-splitting system splits the fluorescence radiation passing through the optical system into at least three parts, each of which forms a respective image of

WO 02/06078

PCT/US02/11003

3

the object corresponding to each of the wavelength regions received. A detector produces a cumulative weighted signal for each image point corresponding to a single point on the object. From the weighted signal values of the various points on the object, an image of the object having improved contrast is produced. This technique is used to aid in distinguishing the fluorescence from the affected tissue from that produced by normal tissue.

A still more complex method of visualizing images from an endoscopic device uses television scanning apparatus. For example, U. S. Patent No. 4,719,508 discloses a method utilizing an endoscopic photographing apparatus wherein the endoscope includes an image sensor for successively generating image signals fed to a first frame memory for storing the image signals and a second frame memory for interlacing and storing image signals read successively from the first frame memory. The stored, interlaced image signals are delivered to a TV monitor for display to aid in visualizing the affected body part.

These prior art endoscopic systems, which rely on photographic processing of the image of the area of interest (i.e., via a TV monitor), while effective, have historically relied on increasingly complex and expensive equipment and substitute image processing to construct a diagnostic image (i.e., indirect viewing) for direct viewing of the affected body part without image processing, as by any type of camera or image processing device.

Certain of the fluorescent dyes that concentrate in tumors due to natural bodily processes can be excited at wavelengths corresponding to those produced by lasers to accomplish diagnostic and therapeutic purposes. Consequently, lasers have also been used in procedures utilizing endoscopic systems in conjunction with fluorescent dyes to image and treat tumors. In one embodiment of this general method, a dye is used that absorbs laser light at two different wavelengths and/or laser powers, one that excites fluorescence without generating damaging heat in the tissue, and one that generates sufficient heat in the dye to destroy surrounding tissue. U.S. Patent No. 4,768,513, for example, discloses a procedure in which a dye is applied to a body part suspected of containing a tumor, usually by local injection. The dye is allowed to

WO 02/080778

PCT/US02/1003

4

concentrate in tumors and clear from healthy tissue over a period of days, and then the body part is irradiated with alternate pulses of two light sources: a white light of a known intensity and a fluorescence-exciting laser light. To compensate for variations in intensity of the fluorescence resulting from variations in the angle of incident light, and the like, visualization of the tumor is computer-enhanced by calculating the intensity of the fluorescence with respect to the known intensity of the white light. Ablation of a tumor detected using this method is accomplished by switching the laser to the heat-generating wavelength so as to destroy the cancerous tissue into which the fluorophore has collected.

While effective for diagnosing and treating tumor, such methods have two major drawbacks. Disease states other than tumor cannot be diagnosed, and laser visualization must be delayed for a period of two days or more after administration of the fluorescent dye to allow the dye to clear from normal tissue.

Monoclonal antibodies and other ligands specific for tumors have been developed for use in diagnosis of tumors, both in tissue samples and in vivo. In addition to such ligands, certain tumor-avid moieties are disproportionately taken up (and optionally or metabolized by tumor cells). Two well-known tumor-avid compounds are deoxyglucose, which plays a telling role in glycolysis in tumor cells, and somatostatin, which binds to and/or is taken up by somatostatin receptors in tumor cells, particularly in endocrine tumors.

In such studies, deoxyglucose is used as a radio-tagged moiety, such as fluorodeoxyglucose (^{18}F -deoxyglucose), for detection of tumors of various types. It is believed that tumor cells experience such a mismatch between glucose consumption and glucose delivery that anaerobic glycolysis must be relied upon, thereby elevating the concentration of the radioactive tag in tumor tissue. It is also a possibility that the elevated concentration of deoxyglucose in malignant tumors may be caused by the presence of isoenzymes of hexokinase with abnormal affinities for native glucose or its analogs (A. Gjedde, Chapter 6: "Glucose Metabolism," *Principles of Nuclear Medicine*, 2nd Ed., W. B. Saunders Company, Philadelphia, PA, pages 54-69). Similarly, due to the concentration of somatostatin in tumor tissue, radio-tagged

WO 02/086778

PCT/US02/11003

5

somatostatin, and fragments or analogs thereof, are used in the art for non-invasive imaging of a variety of tumor types in a procedure known as somatostatin receptor scintigraphy (SRS).

Although these techniques have met with considerable success in determining the presence of tumor tissue, scintigraphic techniques are difficult to apply during a surgical procedure because of the equipment necessary for viewing the image provided by the radioisotope. Yet it is exactly at the time that the surgeon has made the incision or entered the body cavity that it would be most useful to "see" the outlines of the diseased tissue in real time and without the need for expensive and time-consuming image processing equipment.

Thus, there is a need in the art for new and better methods that can be used to directly visualize a broad range of putative disease sites without the need for use of image processing equipment. Where real-time visualization is by means of endoscopic devices, direct visualization (as opposed to images created by image processing equipment) creation of photographic images) offers the additional advantage that the equipment required is comparatively simple to use and is less expensive than the equipment required to process images or create photographic displays from such images. In addition, there is a need in the art for a method of identifying diseased or abnormal tissue during surgical procedures so that immediate resection or biopsy of the identified tissue can be performed while the surgeon "sees" the outlines of the diseased or abnormal tissue.

SUMMARY OF THE INVENTION

The present invention overcomes many of these problems in the art by providing method(s) for *in vivo* identification of diseased tissue in a subject in need thereof. The invention method includes irradiating an *in vivo* body part of the subject containing diseased tissue with light having at least one excitation wavelength in the range from about 401 nm to about 500 nm. Fluorescence emanating from a fluorescent targeting construct administered to the subject and which has specifically bound to and/or been taken up by the diseased tissue in the body part, in response to

WO 02/080778

PCT/US02/11003

6

the at least one excitation wavelength is directly viewed to determine the location and/or surface area of the diseased tissue in the subject.

In another embodiment, the present invention provides methods for utilizing a diagnostic procedure during surgery in a subject in need thereof. In this embodiment of the invention diagnostic methods, an *in vivo* body part of the subject containing diseased tissue is irradiated with light having at least one excitation wavelength in the range from about 401 nm to about 500 nm. A targeting construct preadministered to the subject that fluoresces in response to the at least one excitation wavelength and which has specifically bound to and/or been taken up by the diseased tissue in the body part is directly viewed to determine the location and/or surface area of the diseased tissue in the subject is determined from the directly viewed fluorescence from the targeting construct and at least a portion of the diseased tissue is removed.

In yet another embodiment, the present invention provides methods for *in vivo* diagnosis of tumor tissue in a subject in need thereof. In this embodiment, the invention method includes contacting samples of tumor cells obtained from the subject *in vitro* with a plurality of detectably labeled compounds, each of which binds to or is selectively taken up by a distinct tumor type to determine which of the compounds is bound to or taken up by the sample tumor cells. A biologically compatible fluorescing targeting construct is fabricated to contain a compound determined by this process to bind to and/or be taken up by the sample tumor cells and which fluoresces in response to light having at least one excitation wavelength in the range from about 401 nm to about 500 nm. The location and/or surface area of the tumor tissue in the *in vivo* body part is diagnosed by administering a diagnostically effective amount of the targeting construct to the subject, allowing the targeting construct to bind to or be taken up by *in vivo* tumor cells, and directly viewing fluorescence emanating from the targeting construct bound to or taken up in the tumor tissue in response to irradiation of the tumor tissue with a light that provides the required excitation wavelength.

WO 02/080778

PCT/US02/11003

7

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides method(s) for *in vivo* identification of diseased tissue in a subject in need thereof. The invention method includes irradiating an *in vivo* body part of the subject containing diseased tissue with light having at least one excitation wavelength in the range from about 401 nm to about 500 nm. Fluorescence emanating from a fluorescent targeting construct administered to the subject and which has specifically bound to and/or been taken up by the diseased tissue in the body part, in response to the at least one excitation wavelength is directly viewed to determine the location and/or surface area of the diseased tissue in the subject.

Light having a wavelength range from 401 nm to 500 nm lies within the visible range of the spectrum, in contrast to UV light, which lies within the non-visible range from about 4 nm to about 400 nm. Therefore, the excitation light used in practice of the invention diagnostic methods will contain at least one wavelength of light that illuminates surrounding tissue as well as excites fluorescence from the fluorescent targeting construct used in practice of the invention methods. The excitation light may be monochromatic or polychromatic. To compensate for the tendency of such background effect to obscure the desired diagnostic image, it is preferred to use a filter to screen out wavelengths above about 500 nm in the excitation light, thereby eliminating wavelengths that would be reflected from healthy tissue so as to cause loss of resolution of the fluorescent image. Alternatively, it is possible view the diagnostic site through a filter that substantially screens out wavelengths other than the peak emission wavelength of the fluorophore used. For example, if the fluorescent targeting construct emits fluorescence at a known peak emission wavelength of 520 nm, the filter can be selected to substantially eliminate wavelengths of light other than about 520 nm. Use of a filter in the practice of the invention diagnostic methods is expressly intended to be encompassed by the term "directly viewing" as applied to the invention diagnostic methods.

Use of one or more filters to screen out wavelengths of light in a selected wavelength band or screen out all wavelengths except those in a narrow band is well

WO 02/080778

PCT/US02/11603

8

known in the art and will encompass the use of such simple devices as filtering eyeglasses worn by the diagnostician or physician during the diagnostic procedure. The filter can be a polarizing filter or a non-polarizing filter. For example, a blue filter will generally filter out ultraviolet light as well as visible light in wavelengths longer than the range of blue. Such a filter is particularly useful for observing emittance from fluorophores that fluoresce in the range of blue.

Operating rooms can be equipped with an overhead light that produces wavelengths of light in the optical emitting spectrum useful in practice of invention diagnostic methods, such as a black lamp or a Woods lamp (sometimes referred to as "black-light blue"). Such a light can be utilized in the practice of the invention diagnostic methods merely by turning out the other lights in the operating room (to eliminate extraneous light that would be visibly reflected from tissue in the body part under investigation) and shining the excitation light into the body cavity or surgically created opening so that the fluorescent image received directly by the eye of the observer (e.g., the surgeon) is predominantly the fluorescent image emanating from the fluorophore(s) in the field of vision. Light emanating from a source in the 401-500 nm range could be filtered to aid in accomplishing the goal of direct visualization by the observer so that light reflecting from the body part, other than that from the fluorescing moiety(ies), is minimized or eliminated.

Light in the 401 nm to 500 nm wavelength range is readily absorbed in tissue. Accordingly, in invention diagnostic methods, the diseased tissue (and bound or taken-up targeting construct) is "exposed" to the excitation light (e.g., by surgically created opening or endoscopic delivery of the light to an interior location). The invention method is particularly suited to *in vivo* detection of diseased tissue located at an interior site in the subject, such as within a natural body cavity or a surgically created opening, where the diseased tissue is "in plain view" (i.e., exposed to the human eye) to facilitate a procedure of biopsy or surgical excision. As the precise location and/or surface area of the tumor tissue are readily determined by the invention diagnostic procedure, the invention method is a valuable guide to the

WO 02/080778

PCT/US02/11003

9

surgeon, who needs to "see" in real time the exact outlines, size, etc. of the mass to be resected as the surgery proceeds.

If the putative diseased site is a natural body cavity or surgically produced interior site, an endoscopic device can be optionally used to deliver the excitation light to the site, to receive fluorescence emanating from the site within a body cavity, and to aid in formation of a direct image of the fluorescence from the diseased tissue. For example, a lens in the endoscopic device can be used to focus the detected fluorescence as an aid in formation of the image. As used herein, such endoscope-delivered fluorescence is said to be "directly viewed" by the practitioner and the tissue to which the targeting construct binds or in which it is taken up must be "in plain view" to the endoscope since the light used in the invention diagnostic procedure will not contain wavelengths of light that penetrate tissue, such as wavelengths in the near infra red range. Alternatively, as described above, the excitation light may be directed by any convenient means into a body cavity or surgical opening containing a targeting construct administered as described herein and the fluorescent image so produced can be directly visualized by the eye of the observer without aid from an endoscope. With or without aid from any type of endoscopic device, the fluorescent image produced by the invention method is such that it can be viewed without aid of an image processing device, such as a CCD camera, TV monitor, photon collecting device, and the like.

In one embodiment of the invention diagnostic methods, diseased or abnormal tissue is contemporaneously viewed through a surgical opening to facilitate a procedure of biopsy or surgical excision. As the location and/or surface area of the diseased tissue are readily determined by the invention diagnostic procedure, the invention method is a valuable guide to the surgeon, who needs to know the exact outlines, size, etc. of the mass, for example, for resection as the surgery proceeds.

Accordingly, in this embodiment, the present invention provides methods for utilizing a diagnostic procedure during surgery in a subject in need thereof by irradiating an *in vivo* body part of the subject containing diseased tissue with light having at least one excitation wavelength in the range from about 401 nm to about

WO 02/080778

PCT/US02/11003

10

500 nm, directly viewing fluorescence emanating from a targeting construct administered to the subject that has specifically bound to and/or been taken up by the diseased tissue in the body part, wherein the targeting construct fluoresces in response to the at least one excitation wavelength, determining the location and/or surface area of the diseased tissue in the subject, and removing at least a portion of the tumor tissue.

In yet another embodiment, the present invention provides methods for *in vivo* diagnosis of tumor tissue in a subject in need thereof. In this embodiment, the invention method comprises contacting samples of tumor cells obtained from the subject *in vitro* with a plurality of detectably labeled compounds, each of which binds to or is selectively taken up by a distinct tumor type, determining which of the compounds is bound to or taken up by the sample tumor cells, administering a diagnostically effective amount of at least one biologically compatible fluorescing targeting construct containing a compound determined to bind to and/or be taken up by the sample tumor cells and a fluorophore responsive to at least one wavelength of light in the range from about 401 nm to about 500 nm, and diagnosing the location and/or surface area of the tumor tissue in the *in vivo* body part by directly viewing fluorescence emanating from the targeting construct bound or taken up in the tumor tissue upon irradiation thereof with light providing the at least one excitation wavelength for the fluorescent targeting construct.

In one embodiment of the invention method, a single type of fluorescent moiety is relied upon for generating fluorescence emanating from the irradiated body part (i.e., from the fluorescent targeting construct that binds to or is taken up by diseased tissue). Since certain types of healthy tissue fluoresce naturally, in such a case it is important to select a fluorescent moiety for the targeting construct that has a predominant excitation wavelength that does not contain sufficient wavelengths in the visible range of light to make visible the surrounding healthy tissue and thus inhibit resolution of the diseased tissue. Therefore, the light source used in practice of this embodiment of the invention emits light in the range from about 401 nm to about 500 nm.

WO 02/080778

PCT/US02/11003

11

In alternative embodiments, the invention method may additionally comprise the step of administering to the subject one or more supplemental fluorescing targeting constructs (e.g., antibodies, or biologically active fragments thereof, having attached fluorophores) that bind to the initial fluorescent targeting construct and/or to each other to enhance the fluorescence emanating from the target tissue. For instance, a fluorescently tagged anti-fluorophore antibody may be administered to bind to any previously administered fluorescently-tagged antibody or tumor-avid molecule. The purpose of the supplemental fluorescing targeting construct is to increase the intensity of fluorescence from the targeting ligand of the first administered targeting construct and thereby to aid in detection of diseased or abnormal tissue in the body part.

It is generally good practice to allow the targeting construct to bind to and/or be taken up by any targeting tissue that may be present at the site under investigation and then, before administration of the supplemental fluorescing targeting construct(s), to substantially remove (e.g., wash) from the body part any unbound targeting construct to maximize the opportunity for fluorescence from the supplemental fluorescing targeting construct(s) to aid in detecting the presence of any target tissue present in the body part. Usually, the supplemental fluorescing targeting constructs are successively administered to build up the fluorescent signal from the target tissue. For example, if the fluorescent targeting construct comprises a humanized IgG monoclonal antibody specific for a breast cancer antigen, the next-administered fluorescing targeting construct may comprise an anti-fluorophore antibody, such as anti-fluorescein, and the third-administered fluorescing targeting construct may comprise an anti-idiotypic antibody. Those of skill in the art will be able to devise combinations of successively administered fluorescing targeting constructs, each of which specifically binds to the targeting construct or to one or more of the earlier administered supplemental fluorescing targeting constructs. It is presently preferred that all of the fluorescing targeting constructs used to identify the target tissue comprise fluorophores that fluoresce within the same wavelength band or at the same wave length as does the initially administered targeting construct (e.g. a fluorescing sensitive to the invention wavelength of light in the initially administered targeting construct) to minimize the number of different light sources that need to be employed

WO 02/060775

PCT/US02/1003

12

to excite simultaneous fluorescence from all of the different targeting constructs used in practice of the invention method.

In yet another embodiment, the invention method further comprises the step of administering to the subject at least one supplemental fluorescent targeting construct (e.g., comprising an antibody, or a biologically active fragment thereof having an attached fluorophore) that specifically binds to or is taken up by normal tissue or constructs in the body part, wherein fluorescence from the supplemental fluorescent targeting construct(s) in response to the irradiating light is a different color (i.e., has a different wavelength) than that from the fluorescent targeting construct that is selected to bind to or be taken up by the target tissue. The difference in the colors of the fluorescence emanating from fluorophores in targeting constructs targeted to normal and to diseased or abnormal target tissue aids the observer in determining the location and size of the target tissue. Use of supplemental fluorophores provides the advantage that any natural fluorescence emanating from normal tissue is obscured by the fluorescence emanating from fluorophore(s) in supplemental targeting constructs targeted to the normal tissue in the body part. The greater the difference in color between the fluorescence emanating from normal and target tissue, the easier it is for the observer to visualize the outlines and size of the target tissue. For instance, targeting a fluorescing targeting construct comprising a fluorophore producing red light to the target tissue (i.e., abnormal tissue) and a fluorophore producing green light to healthy tissue aids the observer in distinguishing the target tissue from the normal tissue. Those of skill in the art can readily select a combination of fluorophores that present a distinct visual color contrast.

The spectrum of light used in the practice of the invention method is selected to contain at least one wavelength that corresponds to the predominant excitation wavelength of the targeting construct, or of a biologically compatible fluorescing moiety contained within the targeting construct. Generally the excitation light used in practice of the invention method comprises at least one excitation wavelength of light in the wavelength range from about 401 nm to about 500 nm.

WO 02/060778

PCT/US02/11003

13

However, when a combination of targeting ligands that fluoresce at different wavelengths is used in practice of the invention, the spectrum of the excitation light must be broad enough to provide at least one excitation wavelength for each of the fluorophores used. For example, it is particularly important when fluorophores of different colors are selected to distinguish normal from diseased tissue, that the excitation spectrum of the light(s) include excitation wavelengths for the fluorophores targeted to normal and target tissue.

The fluorescing moiety of the targeting construct or of the supplemental fluorescing targeting ligand(s) can be any chemical or protein moiety that is biologically compatible (e.g., suitable for *in vivo* administration) and which fluoresces in response to excitation light as described herein. Since the targeting ligand is administered to living tissue, biological compatibility includes the lack of substantial toxic effect to the individual in general if administered systemically, or to the target tissue, if administered locally, at the dosage administered. Non limiting examples of fluorophores that can be used in the practice of the invention include fluorescein, mithramycin, and cascade blue, and the like, and combinations of two or more thereof.

Additional non-limiting examples of fluorescent compounds that fluoresce in response to an excitation wavelength in the range from 401 nm to about 500 nm are found in Table 1 below:

TABLE 1

COMPOUND	EXCITATION RANGE (nm)	EMISSION RANGE (nm)
Acridine Red	455-600	560-680
Acridine Yellow	470	550
Acridine	436	520
AFA (Acridine Foulgen SITS)	355-425	460
ACMA	430	474
Astrazon Orange R	470	540
Astrazon Yellow 7 GLL	450	480
Atabrine	436	490
Auramine	460	550
Aurophosphine	450-490	515
Aurophosphine G	450	580
Berberine Sulphate	430	550
DOBO-1, RO-PRO-1	462	481

WO 02/080778

PCT/US02/11003

14

COMPOUND	EXCITATION RANGE (nm)	EMISSION RANGE (nm)
BOPRO 1	462	481
Brilliant Sulpho-flavin PF	430	520
Calcein	494	517
Calcein-AM	440	500-520
Cascade Blue	400	425
Catecholamine	410	470
Chloroacryl	450-490	515
Coryphosphine O	460	575
DIA	456	590
Di-8-ANEPPS	488	605
DiO [DiOC ₁₈ (3)]	484	501
Diphenyl Brilliant Flavin 7GFF	430	520
Eosin	430	540
Fluorescein	494	518
Fluorescein Iso-thiocyanate (FITC)	490	525
Fura 3	485	503
Fura-43	479	588
Fura Red	472 (low [Ca ²⁺]) 436 (high [Ca ²⁺])	657 (low [Ca ²⁺]) 637 (high [Ca ²⁺])
Genacryl Brilliant Yellow 10GF	430	485
Genacryl Pink 3G	470	583
Genacryl Yellow 3GF	430	475
GloXalic Acid	405	460
3-Hydroxypyrene-5,8,10-TriSulphonic Acid	403	513
7-Hydroxy-4-methylcoumarin	360	455
5-Hydroxy-Tryptamine (5-HT)	380-415	520-530
Lucifer Yellow CH	425	528
Lucifer Yellow VS	430	535
LysoSensor Green DND-153, DND-189	442	505
Maxilon Brilliant Flavin 10 GFF	450	495
Maxilon Brilliant Flavin 8 GFF	460	495
MitoTracker Green FM	490	516
Mitochondrin	450	570
NBD	465	535
NBD Amino	450	530
Nitrobenzoxadiazole	460-470	510-650
Nylosan Brilliant Flavin E8G	460	510
Oregon Green 488 fluorophore	496	524
Phosphine 3R	465	565
Quinacrine Mustard	423	503
Rhodamine 110	496	520
Rhodamine 5 GLLD	470	565
Rhodol Green fluorophore	499	525
Sevron Orange	440	530
Sevron Yellow L	430	490
SITS (Prismatene)	395-425	450
Sulpho Rhodamine G Extra	470	570
SYTO Green fluorescent nucleic acid stains	494 ± 6	515 ± 7

WO 02/060778

PCT/US02/11003

15

COMPOUND	EXCITATION RANGE (nm)	EMISSION RANGE (nm)
Thioflavin S	430	550
Thioflavin 5	430	550
Thiozol Orange	453	480
Uranine B	420	520
YOYO-1, YOYO-PRO-1	491	509

Since the fluorescence properties of biologically compatible fluorophores are well known, or can be readily determined by those of skill in the art, the skilled practitioner can readily select a useful fluorophore or useful combination of fluorophores, and match the wavelength(s) of the excitation light to the fluorophore(s). Toxicity of additional useful fluorophores can be determined using animal studies as known in the art.

Preferably, the targeting construct (e.g., the ligand moiety of the invention targeting construct) is selected to bind to and/or be taken up specifically by the target tissue of interest, for example to an antigen or other surface feature contained on or within a cell that characterizes a disease or abnormal state in the target tissue. As in other diagnostic assays, it is desirable for the targeting construct to bind to or be taken up by the target tissue selectively or to an antigen associated with the disease or abnormal state; however, targeting constructs containing ligand moieties that also bind to or are taken up by healthy tissue or cell structures can be used in the practice of the invention method so long as the concentration of the antigen in the target tissue or the affinity of the targeting construct for the target tissue is sufficiently greater than for healthy tissue in the field of vision so that a fluorescent image representing the target tissue can be clearly visualized as distinct from any fluorescence coming from healthy tissue or structures in the field of vision. For example, colon cancer is often characterized by the presence of carcinoembryonic antigen (CEA), yet this antigen is also associated with certain tissues in healthy individuals. However, the concentration of CEA in cancerous colon tissue is often greater than is found in healthy tissue, so an anti-CEA antibody could be used as a ligand moiety in the practice of the invention. In another example, deoxyglucose is taken up and utilized by healthy tissue to varying degrees, yet its metabolism in healthy tissues, except for certain known organs, such as the heart, is substantially lower than in tumor. The

known pattern of deoxyglucose consumption in the body can therefore be used to aid in determination of those areas wherein unexpectedly high uptake of deoxyglucose signals the presence of tumor cells.

In one embodiment according to the present invention, the disease or abnormal state detected by the invention method can be any type characterized by the presence of a known target tissue for which a specific binding ligand is known. For example, various heart conditions are characterized by production of necrotic or ischemic tissue or production of atherosclerotic tissue for which specific binding ligands are known. As another illustrative example, breast cancer is characterized by the production of cancerous tissue identified by monoclonal antibodies to CA15-3, CA19-9, CEA, or HER2/neu. It is contemplated that the target tissue may be characterized by cells that produce either a surface antigen for which a binding ligand is known, or an intracellular marker (i.e. antigen), since many targeting constructs penetrate the cell membrane. Representative disease states that can be identified using the invention method include such various conditions as different types of tumors, bacterial, fungal and viral infections, and the like. As used herein "abnormal" tissue includes precancerous conditions, necrotic or ischemic tissue, and tissue associated with connective tissue diseases, and auto-immune disorders, and the like. Further, examples of the types of target tissue suitable for diagnosis or examination using the invention method include cardiac, breast, ovarian, uterine, lung, endothelial, vascular, gastro-intestinal, colorectal, prostatic tissue, endocrine tissue, and the like, as well as combinations of any two or more thereof.

Representative examples of antigens for some common malignancies and the body locations in which they are commonly found are shown in Table 2 below. Targeting ligands, such as antibodies, for these antigens are known in the art.

WO 02/060778

PCT/US02/1003

17

TABLE 2

ANTIGEN	TUMORS WHERE COMMONLY FOUND
CEA (carcinoembryonic antigen)	colon, breast, lung
PSA (prostate specific antigen)	prostate cancer
CA-125	ovarian cancer
CA 15-3	breast cancer
CA 19-9	breast cancer
HER2/neu	breast cancer
α -feto protein	testicular cancer, hepatic cancer
β -HCG (human chorionic gonadotropin)	testicular cancer, choriocarcinoma
MUC-1	breast cancer
Estrogen receptor	breast cancer, uterine cancer
Progesterone receptor	breast cancer, uterine cancer
EGFr (epidermal growth factor receptor)	bladder cancer

In one embodiment of the invention method, the ligand moiety of the targeting construct is a protein or polypeptide, such as an antibody, or biologically active fragment thereof, preferably a monoclonal antibody. The supplemental fluorescing targeting construct(s) used in practice of the invention method may also be or comprise polyclonal or monoclonal antibodies tagged with a fluorophore. The term "antibody" as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')₂, and Fv that are capable of binding the epitopic determinant. These functional antibody fragments retain some ability to selectively bind with their respective antigen or receptor and are defined as follows:

(1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme pepsin to yield an intact light chain and a portion of one heavy chain;

(2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light

WO 02/060778

PCT/US02/11003

13

chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

(3) (Fab')₂, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')₂ is a dimer of two Fab' fragments held together by two disulfide bonds;

(4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

(5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art. (See for example, Harlow & Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference). As used in this invention, the term "epitope" means any antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

Antibody fragments of the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc

WO 02/060778

PCT/US02/11003

19

fragment directly. These methods are described, for example, by Goldenberg, U.S. patents No. 4,036,945 and No. 4,331,647, and references contained therein, which patents are hereby incorporated in their entireties by reference. See also Nisenzonoff *et al.*, *Arch. Biochem. Biophys.* 89:230, 1960; Porter, *Biochem. J.* 73:119, 1959; Edelman *et al.*, *Methods in Enzymology*, Vol. 1, page 422 Academic Press, 1967; and Coligan *et al.* at sections 2.8.1-2.8.10 and 2.10.1-2.10.4. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of V_H and V_L chains. This association may be noncovalent, as described in Inbar *et al.*, *Proc. Nat'l Acad. Sci. USA* 69:2659, 1972. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. See, e.g., Sandhu, *supra*. Preferably, the Fv fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (scAb) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing scAbs are described, for example, by Whitlow *et al.*, *Methods: a Companion to Methods in Enzymology*, 2: 97, 1991; Bird *et al.*, *Science* 242:423-426, 1988; Pack *et al.*, *BioTechnology* 11:1271-77, 1993; Sandhu, *supra*, and Ladner *et al.*, U.S. patent No. 4,946,778, which is hereby incorporated by reference in its entirety.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells.

WO 02/060778

PCT/US02/11003

20

See, for example, Larrick *et al.*, *Methods: a Companion to Methods in Enzymology*, 2: 106, 1991.

Antibodies which bind to a tumor cell can be prepared using an intact polypeptide or biologically functional fragment containing small peptides of interest as the immunizing antigen. The polypeptide or a peptide used to immunize an animal (derived, for example, from translated cDNA or chemical synthesis) can be conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to the peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid, and the like. The coupled peptide is then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

The preparation of such monoclonal antibodies is conventional. See, for example, Kohler & Milstein, *Nature* 256:495, 1975; Coligan *et al.*, sections 2.5.1-2.6.7; and Harlow *et al.*, in: *Antibodies: a Laboratory Manual*, page 726 (Cold Spring Harbor Pub., 1988), which are hereby incorporated by reference. Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, for example, Coligan *et al.*, sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; *Barney et al.*, Purification of Immunoglobulin G (IgG), in: *Methods in Molecular Biology*, Vol. 10, pages 79-104 (Humana Press, 1992).

Antibodies of the present invention may also be derived from subhuman primate antibodies. General techniques for raising therapeutically useful antibodies in baboons can be found, for example, in *Goldenberg et al.*, International Patent Publication WO 91/11465 (1991) and *Losman et al.*, 1990, *Int. J. Cancer* 46:310,

WO 62/080778

PCT/US02/11003

21

which are hereby incorporated by reference. Alternatively, a therapeutically useful antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al.*, *Proc. Nat'l Acad. Sci. USA* **86**:3833, 1989, which is hereby incorporated in its entirety by reference. Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, *Nature* **321**:522, 1986; Riechmann *et al.*, *Nature* **332**:323, 1988; Verhoeven *et al.*, *Science* **239**:1534, 1988; Carter *et al.*, *Proc. Nat'l Acad. Sci. USA* **89**:4285, 1992; Sandhu, *Crit. Rev. Biotech.* **12**:437, 1992; and Singer *et al.*, *J. Immunol.* **150**:2844, 1993, which are hereby incorporated by reference.

It is also possible to use anti-idiotypic technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the "image" of the epitope bound by the first monoclonal antibody.

In a presently preferred embodiment of the invention method, the ligand moiety in the fluorescent targeting construct used in practice of the invention can be selected from among the many biologically compatible tumor-avid compounds that bind with specificity to receptors and/or are preferentially taken up by tumor cells, and can be used as the ligand moiety in the invention targeting constructs. Tumor-avid compounds that are preferentially "taken up" by tumor cells may enter the cells through surface or nuclear receptors (e.g., hormone receptors), pores, hydrophilic "windows" in the cell lipid bilayer, and the like.

WO 02/080778

PCT/US02/11003

22

Illustrative of this class of tumor-avid compounds are somatostatin, somatostatin receptor-binding peptides, deoxyglucose, methionine, and the like. Particularly useful somatostatin receptor-binding peptides are a long-acting, octapeptide analog of somatostatin, known as octreotide (D-phenylalanyl-L-cysteinyl-L-phenylalanyl-D-tryptophyl-L-lysyl-L-threonyl-N-[2-hydroxy-1-(hydroxymethyl)propyl]-L-cysteinamide cyclic (2,17)-disulfide), lanreotide, an oral formulation of octreotide, P829, P587, and the like. Somatostatin-binding peptides are disclosed in U.S. Patent No. 5,871,711, and methods for linking such peptides covalently to a radioisotope through their carboxyl terminal amino acid under reducing conditions are disclosed in U.S. Patent No. 5,843,401, which are both incorporated herein by reference in their entireties. One of skill in the art can readily adapt such teachings for the preparation of fluorescence-sensitive somatostatin receptor-binding peptides by substituting the fluorescing moieties of this invention in the place of a radioisotope.

Somatostatin and somatostatin receptor-binding peptides are particularly effective for use as the tumor-avid ligand moiety in the targeting construct in the invention diagnostic procedures when the disease state is a neuroendocrine or endocrine tumor. Examples of neuroendocrine tumors that can be diagnosed using the invention method include adenomas (GH-producing and TSH-producing), islet cell tumors, carcinoids, undifferentiated neuroendocrine carcinomas, small cell and non small cell lung cancer, neuroendocrine and/or intermediate cell carcinomas, neuroendocrine tumors of ovary, cervix, endometrium, breast, kidney, larynx, paranasal sinuses, and salivary glands, meningiomas, well differentiated glia-derived tumors, pheochromocytomas, neuroblastomas, ganglioneuro(blasto)mas, paragangliomas, papillary, follicular and medullary carcinomas in thyroid cells, Merkel cell carcinomas, and melanomas, as well as granulomas and lymphomas. These tumor cells are known to have somatostatin receptors and can be targeted using somatostatin or somatostatin receptor binding peptides as the tumor-avid ligand in the invention fluorescent targeting construct.

WO 02/060778

PCT/US02/11003

23

Vasointestinal peptide (VIP), which is used in VIP receptor scintigraphy (L. Virgolini, *Eur. J. Clin. Invest.* 27(10):793-800, 1997, is also useful in the invention method for diagnosis of small primary adenocarcinomas, liver metastases and certain endocrine tumors of the gastrointestinal tract.

Another molecule illustrative of the tumor-avid ligands that are preferentially taken up by tumors is deoxyglucose, which is known to be preferentially taken up in a variety of different types of tumors. Illustrative of the types of tumors that can be detected using deoxyglucose as the tumor-avid ligand moiety in the fluorescent targeting construct as disclosed herein include Preferred tumor targets for deoxyglucose include melanoma, colorectal and pancreatic tumors, lymphoma (both HD and NHL), head and neck tumors, myeloma, cancers of ovary, cancer, breast, and brain (high grade and pituitary adenomas), sarcomas (grade dependent), hepatoma, testicular cancer, thyroid (grade dependent) small cell lung cancer, bladder and uterine cancer, and the like.

Yet other tumor-avid compounds that can be used as the targeting ligand in an invention fluorescing targeting construct are L-amino-cyclobutane-1-carboxylic acid and L-methionine. L-methionine is an essential amino acid that is necessary for protein synthesis. It is known that malignant cells have altered methionine metabolism and require an external source of methionine.

Additional examples of biologically compatible tumor-avid compounds that bind with specificity to tumor receptors and/or are preferentially taken up by tumor cells include mammalian hormones, particularly sex hormones, neurotransmitters, and compounds expressed by tumor cells to communicate with each other that are preferentially taken up by tumor cells, such as novel secreted protein constructs arising from chromosomal aberrations, such as transfers or inversions within the clone.

The term hormone is used herein to refer to compounds that are expressed within a mammal for action at a remote location and includes such compounds as sex hormones, cell growth hormones, cytokines, endocrine hormones, erythropoietin, and

WO 02/086778

PCT/US02/11003

24

the like. As is known in the art, a number of tumor types express receptors for hormones, for example, estrogen, progesterone, androgens, such as testosterone, and the like. Such hormones are preferentially taken up by tumor cells, for example, via specific receptors. It is also known in the art that the particular type of receptors expressed by a tumor cell may change over time with the same cell or cell mass, for example, expressing estrogen receptors at one point in time and with the estrogen receptors being substantially replaced with androgen receptors at another point in time.

Therefore, in another embodiment according to the present invention, the invention diagnostic method comprises prescreening of target tumor cells to determine which receptors are currently being expressed by the target cells. In this embodiment, the invention diagnostic method comprises contacting sample(s) of tumor cells obtained from a subject *in vitro* with a plurality of detectably labeled tumor-avid compounds, and determining which of the tumor-avid compounds bind to or are taken up by the sample cells. The invention diagnostic method further comprises administering to the subject a diagnostically effective amount of one or more biologically compatible fluorescing targeting constructs, each comprising as ligand moiety at least one of the tumor-avid compounds determined to bind to and/or be taken up by the tumor cells so as to allow the fluorescing targeting construct to bind to and/or be taken up selectively *in vivo* by tumor tissue, irradiating an *in vivo* body part of the subject suspected of containing the tumor tissue with light having at least one wavelength in the excitation spectrum of the targeting construct under conditions that substantially eliminates extraneous light to the *in vivo* body part, and directly viewing fluorescence emanating from the fluorescing targeting construct bound to or taken up by the tumor tissue so as to determine the location and/or surface area of the tumor tissue in the *in vivo* body part. Of course, if the tests determine that the tumor cells are concurrently taking up more than one tumor-avid compound in substantial proportion (e.g., both estrogen and progesterone), the more than one tumor-avid compound so determined can be used as the tumor-avid ligand moieties in the targeting constructs in the invention diagnostic method.

W/O 02080778

PCT/US02/1003

25

Methods for obtaining test tumor cells for prescreening to determine the type(s) of tumor-avid compounds that are currently being taken up (e.g., by specific receptors expressed by the tumor cells) are well known in the art. For example, such techniques as fine needle aspirates, scrapings, excisional biopsies, and the like, can in many instances be utilized to obtain test tumor cells relatively non-invasively.

In vitro tests useful for determining the tumor-avid compounds that are being taken up by test tumor cells are numerous and also well known in the art. Such *in vitro* tests generally involve either sequentially or simultaneously contacting the test cells with a plurality of different tumor-avid compounds. For example, the test cells can be contacted with a panel or library of detectably labeled hormones and/or other known tumor-avid compounds to determine which of the detectably labeled compounds bind to and/or are taken up by the test cells.

In the practice of the present invention, the fluorescent moiety sensitive to an excitation wavelength in the 401 nm to 500 nm range can be linked to the tumor-avid compound used as the ligand moiety in the targeting construct by any method presently known in the art for attaching two moieties, so long as the attachment of the linker moiety to the ligand moiety does not substantially impede binding of the targeting construct to the target tissue and/or uptake by the tumor cells, for example, to a receptor on a cell. Those of skill in the art will know how to select a ligand/linker pair that meets this requirement. For example, with regard to octreotide, it has been shown that coupling of a linker to Tyr3 or Phe1 of octreotide does not prevent the internalization of octreotide after binding to the somatostatin receptor (L.J. Hoffman *et al.*, *Proc. Assoc. Am. Physicians* 114:63-9, 1999). It is also known that 1-amino-cyclobutane-1-carboxylic acid can be tagged at the 3 carbon of the ring.

The length of the optional linker moiety is chosen to optimize the kinetics and specificity of ligand binding, including any conformational changes induced by binding of the ligand moiety to a target, such as an antigen or receptor. The linker moiety should be long enough and flexible enough to allow the ligand moiety and the target to freely interact and not so short as to cause steric hindrance between the proteinaceous ligand moiety and the target.

WO 02/080778

PCT/US02/11003

26

In one embodiment, the linker moiety is a heterobifunctional cleavable cross-linker, such as N-succinimidyl (4-iodoacetyl)-aminobenzoate; sulfosuccinimidyl(4-iodoacetyl)-aminobenzoate; 4-succinimidyl-oxycarbonyl-V-(2-pyridyldithio) toluene; sulfosuccinimidyl-6-(α -methyl-V-(pyridyldithio)-toluamido) hexanoate; N-succinimidyl-3-(2-pyridyldithio)-propionate; succinimidyl-6-[3-(2-pyridyldithio)-propionamido] hexanoate; sulfosuccinimidyl-6-[3-(2-pyridyldithio)-propionamido] hexanoate; 3-(2-pyridyldithio)-propionyl hydrazide, Ellman's reagent, dichlorotriazinic acid, S-(2-thiopyridyl)-L-cysteine, and the like. Further bifunctional linking compounds are disclosed in U.S. Patent Nos. 5,349,066, 5,618,528, 4,569,789, 4,952,394, and 5,137,877, each of which is incorporated herein by reference in its entirety.

These chemical linkers can be attached to purified ligands using numerous protocols known in the art, such as those described in Pierce Chemicals "Solutions, Cross-Linking of Proteins: Basic Concepts and Strategies," Seminar #12, Rockford, IL.

In another embodiment presently preferred, the linker moiety is a peptide having from about 2 to about 60 amino acid residues, for example from about 5 to about 40, or from about 10 to about 30 amino acid residues. This alternative is particularly advantageous when the ligand moiety is proteinaceous. For example, the linker moiety can be a flexible spacer amino acid sequence, such as those known in single-chain antibody research. Examples of such known linker moieties include GGGGS (SEQ ID NO:1), (GGGGS)_n (SEQ. ID NO:2), GKSSGSGSESKE (SEQ ID NO:3), GSTSGSGKSSEGKG (SEQ. ID NO:4), GSTSGSGKSSESGSTKG (SEQ ID NO:5), GSTSGSGKSSEGKG (SEQ ID NO:6), GSTSGSGKPGSGEGSTKG (SEQ ID NO:7), EGKSSGSGSESKEF (SEQ ID NO:8), SRSSG (SEQ. ID NO:9), SGSSC (SEQ ID NO:10), and the like. A *Diphtheria* toxin trypsin sensitive linker having the sequence AMGRSGGCAGNRVGSSLSCGHLNLQAM (SEQ ID NO:11) is also useful. Alternatively, the peptide linker moiety can be VM or AM, or have the structure described by the formula: AM(G₁₋₁₁)_nQAM wherein Q is selected from any amino acid and X is an integer from 1 to 11 (SEQ ID NO:12). Additional

WO 02/080778

PCT/US02/11003

27

linking moieties are described, for example, in Huston *et al.*, *PNAS* 85:5879-5883, 1988; Whitlow, M., *et al.*, *Protein Engineering* 6:989-995, 1993; Newton *et al.*, *Biochemistry* 35:545-553, 1996; A. J. Cumber *et al.*, *Bioconj. Chem.* 3:397-401, 1992; Laduner *et al.*, *J. Mol. Biol.* 273:330-337, 1997; and U.S. Patent No. 4,894,443, the latter of which is incorporated herein by reference in its entirety.

The targeting constructs and supplemental targeting constructs used in practice of the invention method can be administered by any route known to those of skill in the art, such as topically, intraarticularly, intracisternally, intracocularly, intraventricularly, intrathecally, intravenously, intramuscularly, intraperitoneally, intradermally, intratracheally, intracavitarily, and the like, as well as by any combination of any two or more thereof.

The most suitable route for administration will vary depending upon the disease state to be treated, or the location of the suspected condition or tumor to be diagnosed. For example, for treatment of inflammatory conditions and various tumors, local administration, including administration by injection directly into the body part to be irradiated by the excitation light (e.g., intracavitarily) provides the advantage that the targeting construct (e.g., fluorescently tagged antibodies) can be administered in a high concentration without risk of the complications that may accompany systemic administration thereof.

The targeting construct is administered in a "diagnostically effective amount." An effective amount is the quantity of a targeting construct necessary to aid in direct visualization of any target tissue located in the body part under investigation in a subject. A "subject" as the term is used herein is contemplated to include any mammal, such as a domesticated pet, farm animal, or zoo animal, but preferably is a human. Amounts effective for diagnostic use will, of course, depend on the size and location of the body part to be investigated, the affinity of the targeting construct for the target tissue, the type of target tissue, as well as the route of administration. Local administration of the targeting construct will typically require a smaller dosage than any mode of systemic administration, although the local concentration of the targeting

WO 02/080778

PCT/US02/11063

28

construct may, in some cases, be higher following local administration than can be achieved with safety upon systemic administration.

Since individual subjects may present a wide variation in severity of symptoms and each targeting construct has its unique diagnostic characteristics, including, affinity of the targeting construct for the target, rate of clearance of the targeting construct by bodily processes, the properties of the fluorophore contained therein, and the like, the skilled practitioner will weigh the factors and vary the dosages accordingly.

The invention composition can also be formulated as a sterile injectable suspension according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1-4, butanediol. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed, including synthetic mono- or diglycerides, fatty acids (including oleic acid), naturally occurring vegetable oils like sesame oil, coconut oil, peanut oil, cottonseed oil, etc., or synthetic fatty vehicles like ethyl oleate, or the like. Buffers, preservatives, antioxidants, and the like, can be incorporated as required, or, alternatively, can comprise the formulation.

The invention fluorescing targeting constructs can be produced by well known techniques. For example, well known techniques of protein synthesis can be used to obtain proteinaceous components of the targeting construct if the amino acid sequence of the component is known, or the sequence can first be determined by well known methods, if necessary. Some of the ligand genes are now commercially available. An advantage of obtaining commercially available genes is that they have generally been optimized for expression in *E. coli*. A polynucleotide encoding a protein, peptide or polypeptide of interest, can be produced using DNA synthesis technology. Methods for obtaining the DNA encoding an unavailable gene and expressing a gene product therefrom are well known and will not be described here in detail.

WO 02/080778

PCT/US02/11003

29

A fluorescent targeting construct comprising a proteinaceous ligand moiety, a proteinaceous linker moiety, and a proteinaceous fluorophore can also be produced as a fusion protein using well known techniques wherein a host cell is transfected with an expression vector containing expression control sequences operably linked to a nucleic acid sequence coding for the expression of the fusion protein (*Molecular Cloning A Laboratory Manual*, Sambrook *et al.*, eds., 2nd Ed., Cold Spring Harbor Laboratory, N.Y., 1989).

"Peptide" and/or "polypeptide" means a polymer in which the monomers are amino acid residues which are joined together through amide bonds, alternatively referred to as a polypeptide. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used, the L-isomers being preferred. Additionally, unnatural amino acids such as beta-alanine, phenylglycine, and homoarginine are meant to be included. Commonly encountered amino acids that are not gene-encoded can also be used in the present invention, although preferred amino acids are those that are encodable. For a general review, see, for example, Spatola, A.F., in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, B. Weinstein, ed., Marcel Dekker, New York, p. 267, 1983.

It will be apparent to those skilled in the art that various changes may be made in the invention without departing from the spirit and scope thereof, and therefore, the invention encompasses embodiments in addition to those specifically disclosed in the specification, but only as indicated in the appended claims.

WO 02/060778

PCT/US02/11003

30

CLAIMS

WHAT IS CLAIMED IS:

1. A method for *in vivo* diagnosis of diseased tissue in a subject in need thereof, said method comprising:

irradiating an *in vivo* body part of the subject containing diseased tissue with light having at least one excitation wavelength in the range from about 401 nm to about 500 nm,

directly viewing fluorescence emitted in response to the light from a fluorescent targeting construct administered to the subject and which has specifically bound to and/or been taken up by the diseased tissue in the body part, and

determining the location and/or surface area of the diseased tissue in the subject from the fluorescence provided by the targeting construct.

2. The method according to claim 1 wherein the light is substantially lacking in wavelengths greater than about 500 nm.

3. The method according to claim 1 wherein the targeting construct further comprises a biologically compatible fluorescing moiety responsive to the excitation wavelength.

4. The method according to claim 1 wherein the ligand moiety is a tumor-avid moiety.

5. The method according to claim 4 wherein the tumor-avid moiety is a hormone, a hormone receptor binding-peptide, deoxyglucose, somatostatin, a somatostatin receptor-binding peptide, or a combination of any two or more thereof.

6. The method according to claim 5 wherein the tumor-avid moiety is somatostatin or a somatostatin receptor-binding peptide.

WO 02/98078

PCT/US02/11003

31

7. The method according to claim 6 wherein the diseased tissue is an neuroendocrine or endocrine tumor.
8. The method according to claim 7 wherein the tumor is melanoma, insulinoma, pancreatic tumors, small cell and non-small cell lung cancer, lymphoma, or ovarian, pituitary, pancreas, or adrenal cancer, brain tumor, colorectal cancer, cutaneous melanoma, epithelial cancer, lung carcinoma, testicular germ cell tumor, or breast cancer.
9. The method according to claim 5 wherein the somatostatin receptor-binding peptide is octreotide, lanreotide, P587 or P829.
10. The method according to claim 4 wherein the tumor-avid moiety is deoxyglucose.
11. The method according to claim 10 wherein diseased tissue is brain tumor, colorectal cancer, lymphoma, cutaneous melanoma, epithelial tumors, lung carcinoma, testicular germ cell tumor, or breast cancer.
12. The method according to claim 4 wherein the tumor-avid moiety is L-amino-cyclobutane-1-carboxylic acid or methionine.
13. The method according to claim 1 wherein the diseased tissue is located in a body opening of the subject and wherein the irradiation of the targeting construct and the direct viewing of fluorescence involves use of an endoscopic device to direct the light to the targeting construct, and to receive fluorescence from the fluorescing targeting construct.
14. The method according to claim 1 wherein the method further comprises surgically excising at least a part of the diseased tissue while directly viewing the fluorescent image.
15. The method according to claim 1 wherein the surface area determined is based on the intensity of the fluorescence.

WO 02/060778

PCT/US02/11003

32

16. The method according to claim 1 wherein the light is substantially monochromatic and the wavelength is matched to a predominant excitation wavelength of the fluorescent targeting construct.
17. The method according to claim 1 wherein a source of the light illuminates at a wavelength below 500 nm.
18. The method according to claim 13 wherein the opening is a natural body cavity.
19. The method according to claim 13 wherein the opening is made surgically.
20. The method according to claim 1 wherein a source of the light is located outside of the body of the subject.
21. The method according to claim 1 wherein the viewing is for monitoring the course of the disease state.
22. The method according to claim 1 wherein the viewing identifies the diseased tissue for surgical intervention.
23. The method according to claim 1 wherein the method further comprises surgically removing at least a portion of the diseased tissue.
24. The method according to claim 1 further comprising administering to the subject at least one supplemental fluorescing targeting construct that binds to the targeting construct to enhance the fluorescence.
25. The method according to claim 24 wherein the at least one supplemental fluorescing targeting construct comprises a monoclonal antibody, or biologically active fragment thereof.
26. The method according to claim 1 wherein the ligand moiety is an antibody or binding fragment thereof.

WO 02/080778

PCT/US02/11003

33

27. The method according to claim 25 further comprising administering to the subject at least one supplemental fluorescing antibody that binds to the fluorescent targeting construct to enhance the fluorescence.

28. The method according to claim 1 wherein the diseased tissue is associated with a condition selected from the group consisting of tumors, bacterial, fungal and viral infections, pre-cancerous conditions, heart attack, stroke, and necrotic and ischemic conditions.

29. The method according to claim 1 further comprising administering to the subject a supplemental fluorescing targeting construct that specifically associates with normal tissue in the *in vivo* body part, wherein fluorescence from the supplemental fluorescing targeting construct in response to the excitation light is a different color than fluorescence from the targeting construct, and wherein the different color distinguishes the tumor tissue from the normal tissue in the body part.

30. The method according to claim 3 wherein the fluorescing targeting construct further comprises a linker moiety for attaching the ligand moiety to the fluorescing moiety.

31. The method of claim 1 wherein the targeting construct is administered by a method selected from the group consisting of topically, intramurally, intracutaneously, intrasclerally, intraventricularly, intrathecally, intravenously, intramuscularly, intravascularly, intercutaneously, intraperitoneally, intradermally, and by a combination of any two or more thereof.

32. The method according to claim 1 wherein the targeting construct is administered by local injection.

33. The method according to claim 1 wherein the targeting construct is administered systemically.

WO 02/080778

PCT/US02/11003

34

34. A method for utilizing a diagnostic procedure during surgery in a subject in need thereof, said method comprising:

irradiating an *in vivo* body part of the subject containing diseased tissue with light having at least one excitation wavelength in the range from about 401 nm to about 500 nm,

directly viewing fluorescence emanating from a fluorescent targeting construct administered to the subject and which has specifically bound to and/or been taken up by the diseased tissue in the body part, wherein the targeting construct fluoresces in response to the at least one excitation wavelength,

determining the location and/or surface area of the diseased tissue in the subject from the directly viewed fluorescence from the targeting construct, and

removing at least a portion of the diseased tissue.

35. The method according to claim 34 wherein the excitation light is substantially lacking in light having a wavelength greater than about 500 nm.

36. The method according to claim 34 wherein the viewing of the fluorescence and the removing of the tumor tissue are performed substantially contemporaneously.

37. The method according to claim 34 wherein the targeting construct further comprises a biologically compatible fluorescing moiety linked to the tumor-avid moiety.

38. The method according to claim 37 wherein the tumor-avid moiety is a hormone, deoxyglucose, somatostatin, a somatostatin receptor-binding peptide, or a combination of any two or more thereof.

WO 02/080778

PCT/US02/11003

35

39. A method for *in vivo* diagnosis of tumor cells in a subject in need thereof, said method comprising:

(a) contacting samples of tumor cells obtained from the subject *in vitro* with a plurality of detectably labeled compounds, each of which binds to or is selectively taken up by a distinct tumor type,

(b) determining which of the compounds is bound to or taken up by the sample tumor cells,

(c) administering a diagnostically effective amount of at least one biologically compatible fluorescing targeting construct containing a compound determined in (b) to bind to and/or be taken up by the sample tumor cells and which fluoresces in response to light having at least one excitation wavelength in the range from about 401 nm to about 500 nm, and

(d) diagnosing the location and/or surface area of the tumor tissue in the *in vivo* body part by directly viewing fluorescence emanating from the targeting construct bound to or taken up in the tumor tissue in response to irradiation of the tumor tissue with the light.

【国際調査報告】

INTERNATIONAL SEARCH REPORT		International application No. PCT/US06/11090
A. CLASSIFICATION OF SUBJECT MATTER IPC7 : A61B 10/00 US CL : 4249.6 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELD OF SEARCH Minimum documentation searched (classification system followed by classification symbols) U.S. : 4249.11, 5.1, 9.6 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base used, where practicable, search terms used) USPAT-FULL, BIOSIS, MEDLINE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of documents, with indication, where appropriate, of the relevant passages U.S. 6,254,213 B1 (LUSKIN) 04 September 2001 (04.09.2001), non-patent literature, especially the abstract; column 3, line 58 - column 4, line 44; and column 19-24, claims 1-46. 1-99	Relevant to claim No.
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" documents defining the general state of the art which is not considered to be of particular relevance "X" earlier applications or patents published on or after the international filing date "Y" documents which may throw doubts on priority claims or which is cited to establish the prior art date of earlier claims or other special interest (as specified) "Z" documents referring to an oral disclosure, use, exhibition or other means "P" documents published prior to the international filing date but later than the priority date defined		
"W" documents published after the international filing date or priority date and in conflict with the application but cited to support the priority or novelty of the invention "Q" documents of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "R" documents of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is considered in context, or together with other such documents, such combination being obvious to a person skilled in the art "S" documents members of the same patent family		
Date of the actual completion of the international search 31 May 2002 (31.05.2002)		Date of mailing of the international search report 10 JUN 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20531 Facsimile No. (703) 305-3230		Authorized officer D. L. Arney <i>Teresa D. Roberts</i> Telephone No. (703) 506-1735

Form PCT/ISA/210 (second sheet) (July 1998)

フロントページの続き

(81)指定国 AP(GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZM,ZW),EA(AM,AZ,BY,KG,KZ,MD,RU,TJ,TM),EP(AT, BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE,TR),OA(BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GM,ML,MR,NE,SN, TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EC,EE,ES,FI,GB,GD,GE, GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,ME,MK,MN,MW,MX,MZ,NO,NZ,OM,PH,P L,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TN,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZM,ZW

Fターム(参考) 2G043 AA03 BA16 CA05 DA02 EA01 FA01 KA02 KA05 LA01 LA03

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record.**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☒ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☒ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.